The Use of Real-Time Polymerase Chain Reaction in Quantifying Inflammatory Responses to Biomaterials

LeeAnn O. Bailey, Newell R. Washburn, Francis W. Wang
Biomaterials Group, Polymers Division, National Institute of Standards and Technology, Gaithersburg, MD. 20899-8545

Introduction: The introduction of tissue engineering approaches for the repair and replacement of human body components extends the application and importance of synthetic materials (biomaterials). Implanted biomaterials frequently evoke inflammatory responses, which are subsequently responsible for implant failure. The molecular mechanisms and the signal transduction that occurs during inflammatory responses are quite complex. Furthermore, the mechanisms regarding the initiation and propagation of inflammatory responses are not well defined at present. To evaluate the biocompatibility of materials, we have established a system that allows for the analysis and quantitation of cellular inflammatory responses *in vitro*. Elevated cytokine production serves as an indicator of inflammatory responses, thus we monitored the levels of interlukin-1β produced by the cells. In this study, the inflammatory responses of non-transformed murine macrophages (Raw 264.7) cells were analyzed after incubation with polymethylmethacrylate (PMMA) microspheres in the presence and absence of lipopolysaccharide (LPS) for 8 h. The analysis of the genetic material obtained from the macrophages was quantitated using Real-Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). RT-PCR can be used to rapidly and quantitatively analyze several gene products from multiple small samples simultaneously. The goals of this work are to develop improved measurement methods for the quantification of cellular inflammatory responses to biomaterials and to obtain data that culminates in an enhanced understanding of the ways in which the body responds to the introduction of biomaterials.

Experimental:

RAW 267.4 cells, were maintained in RPMI medium 1640 (Life Technologies) supplemented with 10 % (volume fraction) heat inactivated fetal bovine serum in 5 % CO_2:95 % air at 37 °C. To harvest, cells were washed with calcium and magnesium free phosphate buffered saline, and subsequently incubated with Hank's buffered salt solution to promote release from the flask.

Treatment

Raw 264.7 cells were plated and allowed to reach 70 % confluency in a 75 cm^2 flask. After reaching confluency, .01 mL of a polymethylmethacrylate (PMMA) microsphere suspension (1.915×10^9 /mL) (Bangs Laboratories) was added to the culture. Lipopolysaccharide (1µg/mL was added after 24 h and allowed to incubated in the culture for 8 h.

mRNA Harvest

The messenger RNA extraction from sample cell populations was carried out using the materials and protocol provided in the Rneasy Kit from Qiagen. The RNA was normalized using spectrophotometric techniques and densitometry, so that the starting quantity of RNA was equivalent in each RT-PCR reaction.

RT-PCR

Primers were designed using Primerfinder (Whitehead Institute for Biomedical Research) to design primers for RT-PCR experiments. The primers generated were used in PCR and RT-PCR experiments. They are as follows: 18S ribosomal subunit: 5' agcgaccaaaggaaccataa 3' and 3' ctcctcctcctcctctctcg 5'

Interleukin-1 beta: 5' tgtgaaatgccacctttga 3' and 3' gtagctgccacagcttctcc 5'

The amplicons generated from these primers are 204 base pairs, and 205 base pairs respectively. RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit and protocol (Qiagen), and ribosomal subunit 18 was used as a control.

Results: Populations of Raw 264.7 cells were harvested and analyzed using RT-PCR. Figure 1 displays a plot that shows the gene copy number quantitated from each of the cell samples. Raw 264.7 cells were also allowed to incubate with PMMA particles alone, however there was no detectable copy number above control levels (data not shown).

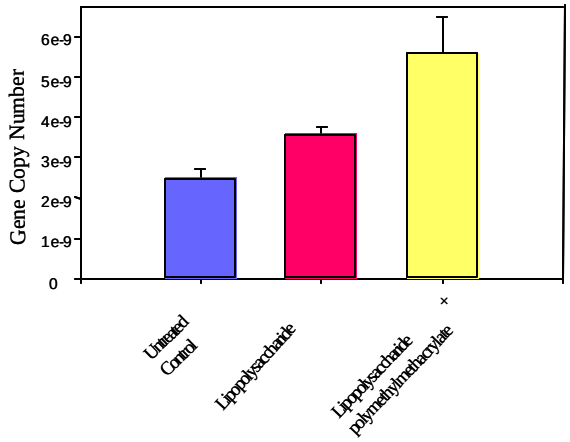

Figure 1. Gene copy number of interleukin-1β after 8 h of treatment. Error bars are representative of one standard deviation from the mean of triplicate samples harvested from a single population of Raw 264.7 cells, and is the estimate of the standard uncertainty.

The cell population treated with LPS alone resulted in a 1.2 fold induction, while cells incubated with LPS and PMMA particles showed a 2.5 fold induction of the cytokine. Thus, we have demonstrated that the presence of a bacterial component and wear debris particles can work in concert to elevate cytokine production. Several cytokines are known to play prominent roles in propagating and mediating inflammatory responses, and we are currently investigating other mediators using RT-PCR. In addition, RT-PCR and its application are also relevant to other systems, and research is currently underway in several areas.

***Disclaimer**: Certain commercial materials and equipment are identified in this article to specify the experimental procedure. In no instance does such identification imply recommendation or endorsement by the National Institute of Standards and Technology or that of the material or equipment identified is necessarily the best available for the purpose.